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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

NKX3.1 is a homeoprotein with prostate-specific expression in adult mammals. *NKX3.1* gene targeting studies in mice have shown that the protein is a differentiating factor and suppressor of epithelial cell growth. Importantly, haploinsufficiency in the mice was dominant, suggesting that altered levels of protein could impart a phenotype in humans. We have described a genetic polymorphism, C154T, in 10% of the population that alters the amino acid sequence of the protein from arginine to cysteine at amino acid 52 (R52C). The R52C variation results in a 70% decrease in in vitro phosphorylation of NKX3.1 at serine 48. Moreover, whereas DNA binding of wild type protein is regulated by phosphorylation, binding of NKX3.1 R52C to DNA is not affected by phosphorylation. Heterozygosity for *NKX3.1* C154T is a risk factor for aggressive prostate cancer (RR=1.8). NKX3.1 appears to suppress transcription when it alone binds to DNA. However, NKX3.1 has been shown to activate transcription by interacting with known transcription factors, such as serum response factor. We have identified regions of NKX3.1 that regulate transcriptional coactivation. In addition we have identified a new biochemical assay for NKX3.1 activity by virtue of its interaction with the CMV early region promoter. Lastly, we have preliminarily isolated other proteins that bind to NKX3.1.

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INTRODUCTION

NKX3.1 is a prostate-specific homeobox gene that maps to a region of chromosome 8p21 that is lost in up to 85% of prostate cancer cases. Although NKX3.1 does not undergo somatic mutations in prostate cancer, loss of expression with tumor progression suggests a role for NKX3.1 in prostate cancer pathogenesis. In NKX3.1+/- mice haploinsufficiency is dominant, resulting in prostatic epithelial hyperplasia and dysplasia that worsen with age. Moreover, NKX3.1 haploinsufficiency cooperates with loss of other suppressor genes such as PTEN to enhance prostate carcinogenesis. These data suggest that loss of NKX3.1 expression may be important in pathogensis of a large fraction of human prostate cancers and that NKX3.1 is a candidate gatekeeper gene. We described an NKX3.1 polymorphism, C154T that resulted in an arginine to cysteine alteration of codon 52 (NKX3.1 R52C) [1]. We showed that a single NKX3.1 C154T allele, present in 11% of the population, conferred an increased risk for aggressive prostate cancer. We also showed that the R52C variant altered phosphorylation at the adjacent serine 48 (S48). Phosphorylation at serine 48 regulated DNA binding in vitro [2]. The current project aims to determine mechanisms of action of NKX3.1 and to understand the effects of the R52C polymorphism on protein function.

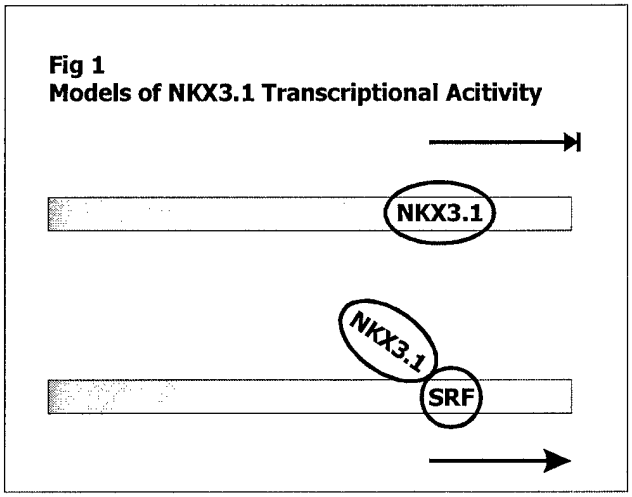


Fig 1
Models of NKX3.1 Transcriptional Acitivity

Using affinity purification chromatography we identified the hexanucleotide high-affinity DNA binding sequence for NKX3.1 – TAAGTA [3]. When these sequences were placed upstream from a minimal HSV TK promoter in a luciferase reporter construct we found that expression of NKX3.1 resulted in suppression of reporter activity [3]. In fact we have routinely found that insertion of the high-affinity DNA binding site for NKX3.1 upstream from promoters results in NKX3.1-dependent suppression of transcription. For example, when we investigated the effect of NKX3.1 on androgen-driven transcription using the MMTV LTR steroid-response element in a reporter construct, we found that the MMTV-LTR, which contains one consensus and several near consensus NKX3.1 binding sites, was suppressed by NKX3.1 (our unpublished observations). These finding are in contrast to the activity of NKX2.5, which stimulates a minimal promoter placed downstream from a triplet consensus NKX2.5 DNA binding sequence [4].

Because of some similarities in the expression of NKX3.1 and NKX2.5 during early stages of murine development, Carson et al examined the effect of NKX3.1 on the activity of the chicken smooth muscle γ-actin (SMGA) promoter [5]. SMGA is expressed during somitogenesis and has been shown to be controlled by NKX2.5 which acts as a transcriptional coactivator for SMGA by binding to SRF [4]. Binding of NKX2.5 to SRF is DNA-independent. In this manner, NKX2.5 acts as a transcriptional coactivator for SRF and will augment the effect of SRF on promoters that contain SRE motifs, the DNA binding regions for SRF. Similarly, Carson found that NKX3.1 bound to SRF and enhanced activation of the SMGA promoter in reporter gene assays [5]. NKX3.1 can also inhibit the activity of transcription factors. Recently Chuck Bieberich's lab isolated prostate-derived ETS-factor, (PEDF) by a yeast two-hybrid screening using Nkx3.1 as bait. In reported gene assays Nkx3.1 was found to repress transcriptional activation by PDEF [6]. These data led us to generate the model for NKX3.1 action shown in Figure 1. When NKX3.1 binds to high-affinity DNA binding

sites it inhibits transcription. NKX3.1 can also bind to serve as a coactivator for other transcription factors and thereby can activate or repress gene expression.

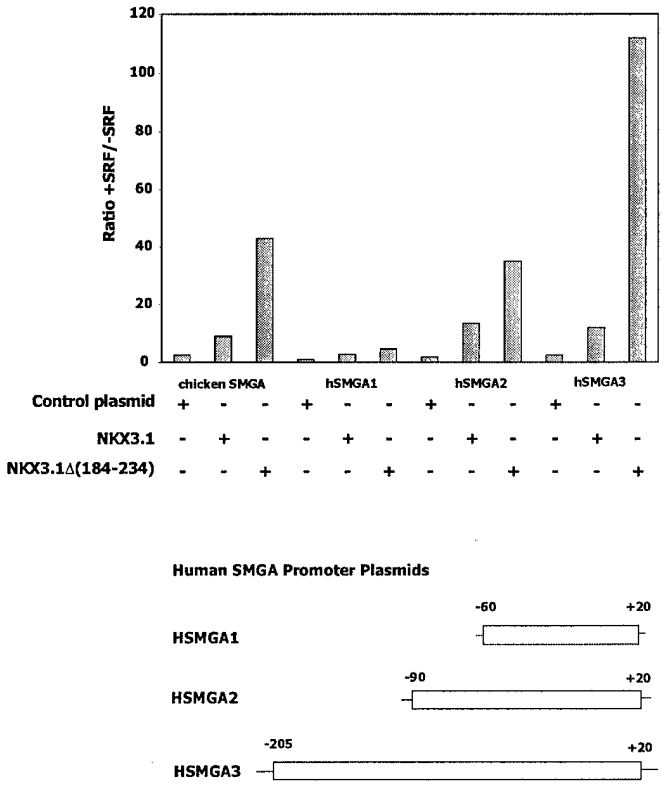
BODY
(Organized according to the Statement of Work)

A. Construction and testing of NKX3.1 reporter genes with wild-type and mutant *NKX3.1* expression vectors.

1. Testing of human NKX3.1 effects on chicken SMGA reporter plasmid

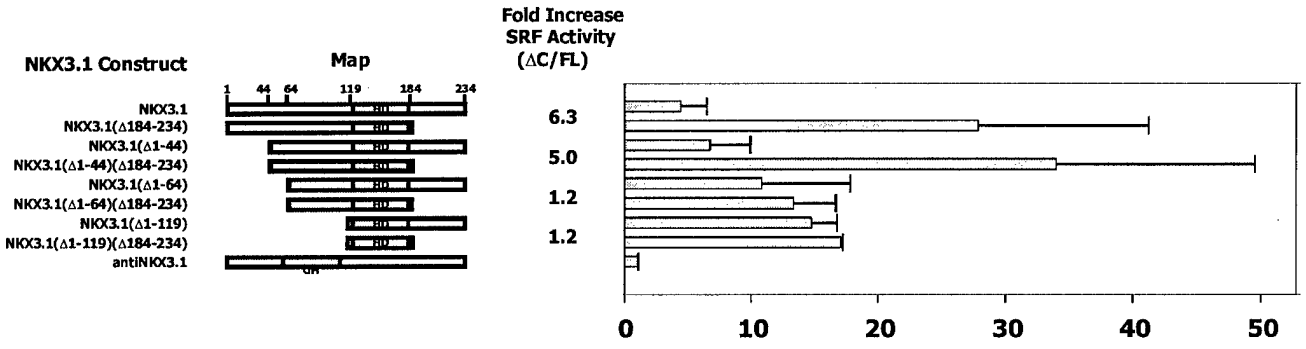
We wanted to determine whether human NKX3.1 reacted similarly with SRF as murine Nkx3.1. We employed the chicken SMGA promoter construct and three reporter plasmids that contained fragments of the human SMGA promoter. The data in Figure 2 show that the interaction of human NKX3.1 with SRF activated chicken and human SMGA constructs to similar degrees. The C-terminal truncated NKX3.1Δ(184-234) had a greater effect on the longest human promoter fragment than on the chicken SMGA promoter (Figure 2).

Fig 2
Analysis of Human Smooth Muscle γ-Actin Promoter



The increase in SRF coactivation caused by deletion of the C-terminal domain of NKX3.1 is similar to the activation of NKX2.5 caused by deletion of that C-terminal domain [7]. Since the sequences of the C-terminal domains of NKX3.1 and NKX2.5 are largely different, even to the degree that NKX3.1 lacks a classical NK2 domain in the C-terminus, we conducted a deletion analysis of NKX3.1 to understand better the regulation of its binding to SRF. For the reporter assays CV-1 fibroblasts were maintained in Modified Improved MEM (Life Technologies, Inc.) supplemented with 5% fetal bovine serum. Cells were plated at ~1-2 X10^5 cells/well in 12 well plates. Cells were transfected 24 hours after plating, using Lipofectamine Plus according to manufacturer's protocol (Life Technologies, Inc.). Each transfection reaction contained either 0.25μg of chicken SMGA reporter plasmid or 0.25 μg human SMGA reporter plasmid clone and either 0.2μg NKX3.1 or 0.2μg NKX3.1(Δ184-234). 0.5μg SRF expression plasmid was used as indicated. Total DNA transfected was always kept the same and balanced to 0.5μg with empty vector. Cells were lysed 24 hours after transfection, and the lysate was assayed for firefly luciferase activities with Dual Luciferase Reporter Assay Reagents (Promega, Madison, WI). In SRF coactivation assays we analyzed the ability of the constructs to increase expression from the SMGA promoter in the presence of SRF. Results of parallel SRF coactivation assays are shown in Figure 3. The results shown in the histogram are normalized to the control inverted (anti) *NKX3.1* plasmid plus SRF and expressed as fold-increase over that background level. The anti-NKX3.1 construct is not transcribed and is used as a negative

Fig 3
Effect of NKX3.1 Deletions on SRF Coactivation



control. The table to the left of the histogram shows the ratio of activity of C-terminal truncated construct to the corresponding construct with an intact C-terminal domain. Determinations were done at least three and up to ten times. Note that C-terminal deletion of the full-length and NKX3.1(Δ1-44) constructs increases interaction with SRF 6.3- and 5-fold, respectively. However, the NKX3.1(Δ1-64) construct was not affected by C-terminal deletion (1.2-fold difference in activity). The activity of the NKX3.1(Δ1-64) construct is 2.5 times as active as the full-length construct. This suggests that the N-terminal region between amino acids 44 and 64 was, in part, responsible for the inhibition exerted by the C-terminal region. This is supported by the observation that the NKX3.1(Δ1-64) and NKX3.1(Δ1-64)(Δ184-234) constructs had comparable activity. A similar lack of C-terminal inhibition was seen with the NKX3.1(Δ1-119) construct. Note that the construct containing only the 64 amino acids from 120-183 included the entire 60-amino acid homeodomain. The localization of SRF coactivation in the homeodomain of NKX3.1 is similar to findings with NKX2.5 [4].

2. Construction of NKX3.1 and SRF expression vectors under control of HSVtk promoter

Because NKX3.1 affected the activity of the CMV promoter in the pcDNA3 expression vector used to express NKX3.1, we wanted to engineer an NKX3.1 expression vector that was controlled by a different promoter such as the *Herpes simplex* virus thymidine kinase promoter (HSVtk). We utilized a number of subcloning strategies to generate this construct. Unfortunately, after numerous attempts we never derived a single plasmid that contained the desired sequences. Our subcloning strategy included the using of both simultaneous and internal controls for restriction enzyme digestion and ligation. We concluded that NKX3.1 expression driven by an HSVtk promoter was a forbidden construct in *E. coli* and we have abandoned this task as not feasible.

To generate an expression plasmid of NKX3.1 driven by the HSVtk promoter we digested the plasmid pRL-TK with two different restriction enzymes and digested *NKX3.1* cDNA in pcDNA3.1 with the same two enzymes to generate cohesive sticky ends. We analyzed the restriction digest products on agarose gel and isolated the *NKX3.1* and promoter-containing fragment of pRL-TK fragments via Rapid Gel Extraction. The restriction enzymes Nhe I and Xba I were used to digest the DNA and generate cohesive ends as were the enzymes Hind III and Xba I. Digestions were done in tandem after which DNA was run on the gel to see if enzyme was functioning properly and that the digest went to completion. After one digest, we purified the DNA and then digested using the other enzyme. Double digests were also attempted when possible. The fidelity of the enzymes was also tested on other known DNA fragments. We then ligated the NKX3.1 insert with TK promoter-containing vector using T4 DNA ligase, transformed competent bacteria, and analyzed plasmids from antibiotic-resistant bacterial colonies.

During ligation we tried many different ratios of vector to insert. We also ran the ligation reaction mixture +/- DNA ligase on agarose gels to compare DNA band patterns. We obtained results showing differences in the two lanes, indicating that a ligation could have successfully occurred. Various amounts of ligation product was transformed into DH5α competent *E.coli*, Maximum Efficiency bacteria. Most of the time, we did not produce any antibiotic-resistant colonies. If there were colonies, it was proved to not be the desired product after analysis. We repeated the cloning attempts with *NKX3.1*Δ(1-119) and *NKX3.1*Δ(184-234) without any success.

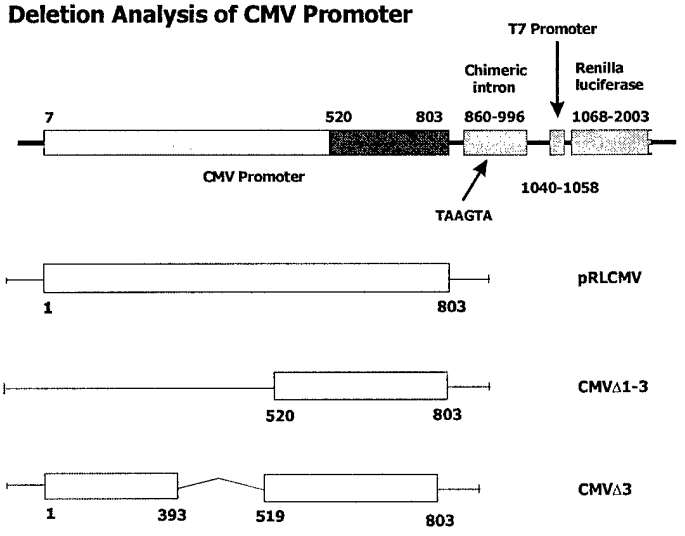
In another subcloning strategy we digested the promoter-containing and *NKX3.*1-containing plasmids and bypassed gel purification of the inserts by adding all four fragments to the ligation mixture. Colonies were isolated and found only to contain the parental plasmids and not the TK-*NKX3.*1 construct. Southern-type hybridization of resultant colonies failed to identify any colonies that contained both TK and *NKX3.1* sequences.

As a result of these attempts we are recommending that this element of the Statement of Work be deleted and replaced by expression experiments with pcDNA3.1 constructs performed in different host cells. We have found that host cell influenced the interaction between the NKX3.1 protein and the CMV promoter. In TSU-Pr1 bladder cancer cells there was a profound difference in activity of the CMV promoter-driven NKX3.1 expression constructs depending on the presence or absence of the C-terminal domain of NKX3.1. In contrast, this difference was markedly attenuate when the expression plasmids were introduced into CV-1 cells. Additional experiments will pursue the goals of this Statement of Work element in CV-1 cells. If the DOD agrees with this recommendation, the results of these experiments will be provided with the next progress report.

3. Deletion analysis of CMV promoter

We stated in our proposal that *NKX3.1*Δ(184-234) was expressed from a pcDNA3.1 expression plasmid at a much higher level than *NKX3.1* in TSU-Pr1 cells. We felt that this may have been due to interaction of NKX3.1Δ(184-234) with the CMV promoter driving transcription in the pcDNA3.1 expression vector. Since this observation was thought to provide a clue to a possible NKX3.1 reporter construct, we analyzed the CMV promoter by deletion analysis. The CMV promoter construct in the *Renilla* luciferase reporter was cleaved by restriction enzyme digestion and religated as shown in Figure 4. The two deletion constructs were tested as reporters with NKX3.1 and NKX3.1Δ(184-234). As shown in Figure 5 all the reporter activity was confined to the 3' 284 nucleotides. We did note that the chimeric intron upstream from the T7 polymerase promoter in the reporter construct contained a perfect TAAGTA NKX3.1 binding site. However, mutation of this site to a nonbonding hexanucleotide did not alter the responsiveness of the CMVΔ3 construct to with NKX3.1 or NKX3.1(184-234) [3]. Sequence analysis of the CMV promoter fragment in CMVΔ3 did not reveal any SRF response elements.

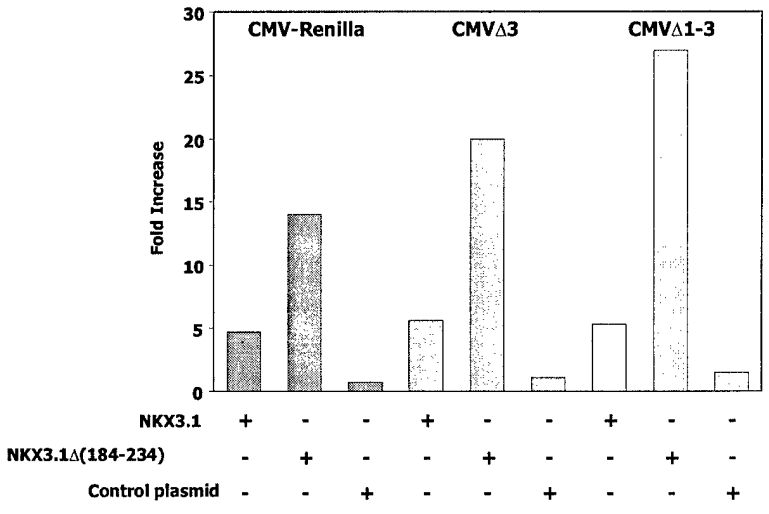
Fig 4
Deletion Analysis of CMV Promoter



4. Analysis of NKX3.1 WT and mutant constructs driven by HSVtk on CMV and SMGA vectors

Because the HSVtk-driven reporter constructs could not be constructed we were unable to complete this element of the Statement of Work. We propose to replace this element with analysis of NKX3.1 and NKX3.1Δ(184-234) as well as other deletion constructs in CV-1 and TSU-Pr1 cells to compare the activities of the proteins and help determine the elements that control activity of the protein.

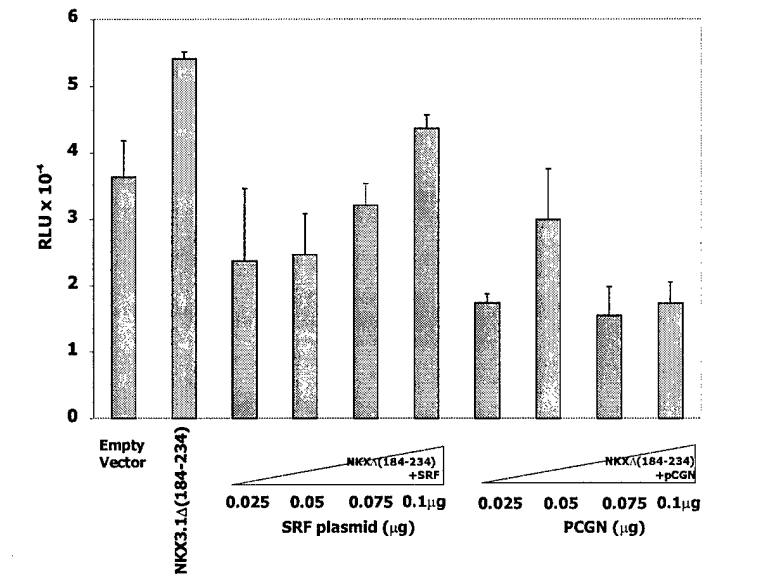
Fig 5
Effect of NKX3.1 on CMV Promoter Constructs



5. Effect of SRF on NKX3.1 interactions with CMV-derived reporter plasmids

To determine whether the responsiveness of CMVΔ3 was due to an interaction between NKX3.1Δ(183-234) and SRF we titrated exogenous SRF expression plasmid and a control empty expression vector in a CMVΔ3 reporter assay. The data are shown in Figure 6. Empty SRF expression plasmid alone had a squelching effect on the interaction between NKX3.1Δ(184-234) and CMVΔ3 promoter. SRF at higher plasmid levels overcame this squelching to some degree, but did not restore the full interaction between NKX3.1Δ(184-234) and CMVΔ3.

Fig 6
Effect of SRF on Interaction of NKX3.1Δ(184-234) with CMVΔ3



Further experiments will be required to determine the exact role of SRF in the interaction of NKX3.1Δ(184-234) and the CMVΔ3 fragment since by sequence analysis we know that CMVΔ3 does not contain a consensus SRE, the DNA binding site for SRF. These data suggested that NKX3.1Δ(184-234) interacts with the CMVΔ3 promoter fragment by coactivation.

In the next set of experiments we asked whether homeodomain mutations known to disrupt DNA binding of NKX3.1 affected the interaction with CMVΔ3. In Figure 7 we showed that mutations T179A and N174Q located in the DNA-binding helix 3 of the proein and disrupt DNA binding had no effect on the interaction of NKX3.1 with CMVΔ3 or the full-length CMV promoter. Because the CMV promoter is substantially more sensitive to NKX3.1Δ(184-234) we also introduced these two homeodomain helix 3 mutations into C-terminal truncated NKX3.1. In addition we introduced L139S, a helix 1 mutation that changes a leucine residue conserved in all homeodomain proteins. Lastly, we tested constructs that affected the S48 phosphorylation site including the R52C polymorphism [2], S48A that abrogated phosphorylation, and S48E that was introduced to mimic constitutive phosphorylation. Mutations affecting phosphorylation had small affects on the interaction of C-terminal truncated NKX3.1 with CMVΔ3 (Figure 8). One DNA-binding mutation, T179A had no effect on the interaction, but two disrupted the interaction. The preliminary conclusion, pending DNA binding analysis, is that DNA binding of NKX3.1Δ(184-234) is not required for the CMVΔ3 interaction, but that homeodomain mutations can disrupt protein-protein interactions that affect the interaction. DNA binding experiments are underway to confirm this preliminary conclusion. It should also be noted that NKX3.1Δ(184-234)(N174Q) is active in SRF coactivation and is twice as potent as NKX3.1(N174Q). For this reason we do not believe that the effect of NKX3.1 on the CMVΔ(1-3) promoter fragment is mediated by SRF.

Fig 7
Effect of Homeodomain Mutations on NKX3.1 Interaction with CMV Promoter

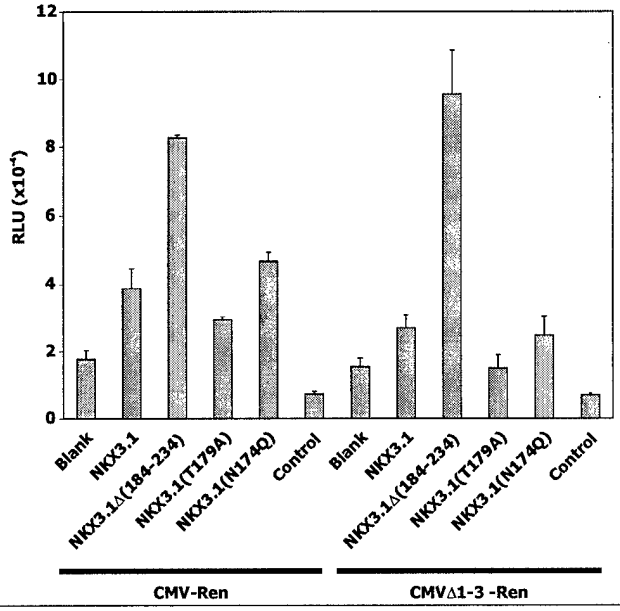
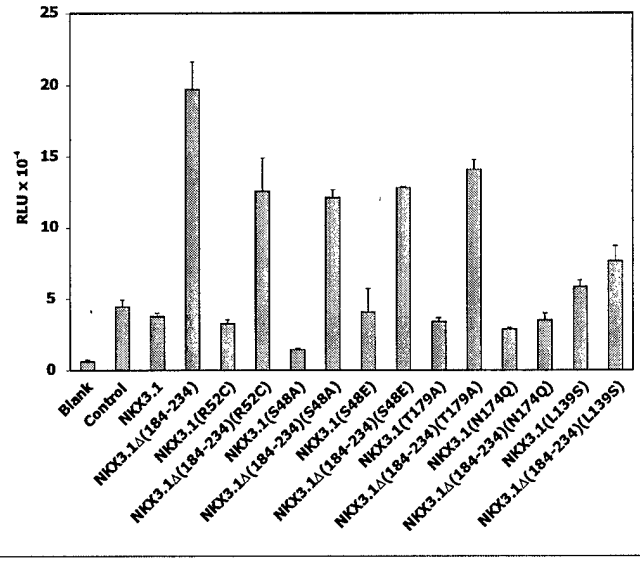


Fig 8
Effect of Homeodomain Mutations on CMV Promoter Interaction



B. Yeast-two-hybrid cloning of NKX3.1 binding partners. (Year 1-2)

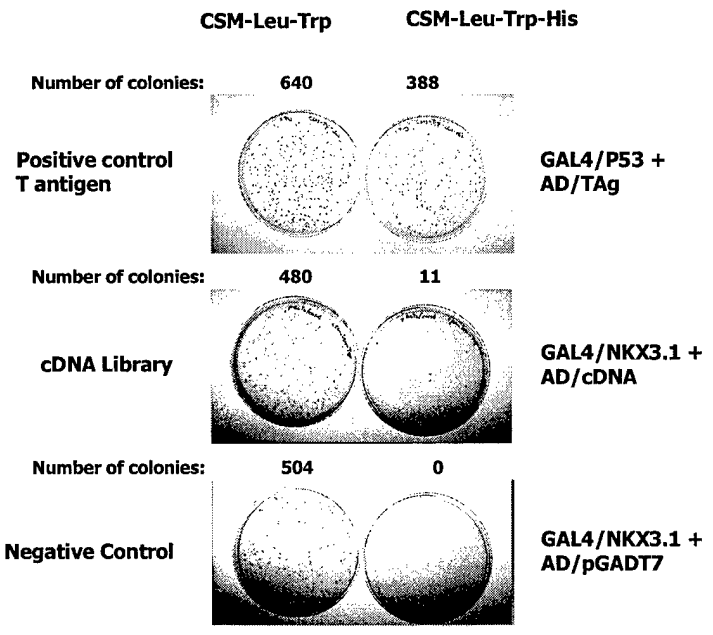
To improve our understanding of the mechanism of NKX3.1 action we wanted to identify proteins that bind to the NKX3.1. We carried out yeast two-hybrid selection of proteins that bind to full-length NKX3.1. Our preliminary findings have raised the possibility that NKX3.1 works in part by a mechanism we had not anticipated – by binding to metabolic enzymes. We used the Matchmaker III® yeast two-hybrid selection system. NKX3.1, fused to yeast GAL4 DNA-binding domain (DBD), was employed as "bait". We screened a cDNA library made from LNCaP prostate

cancer cells and a second library made from human kidney cDNA both fused to the yeast GAL4 activation domain (AD). The LNCaP library was constructed in our lab and was made from size-selected cDNA and shown to have a large fraction of plasmids with inserts in the range from 800-2.5 kb. The human kidney library was obtained from Clonetech. The yeast two-hybrid bait vector was shown by in vitro transcription/translation reaction to code for immunoreactive NKX3.1 and was shown to produce a fusion protein of NKX3.1 and the GAL4 DNA-binding domain. Yeast two-hybrid screening of the two cDNA libraries fused to the GAL4 activation domain (AD) is shown schematically in Figure 8.

To test for transformation efficiency library was plated on Leu$^-$, Trp$^-$ medium, that selects for expression of both the bait and the AD/library plasmid, but not for the two-hybrid interaction directly. As shown in Figure 9, both control constructs and library constructs (cDNA/AD) plated with equal efficiency on Leu$^-$,Trp$^-$ plates. The expression of HIS3 and growth in His$^-$ medium selects for yeast two-hybrid interaction. Figure 9 shows that the positive control, interaction of P53 and SV40 T antigen, gave a large number of HIS+ colonies. The negative control (Figure 9 bottom) gave no HIS+ colonies. The library gave a few colonies, indicative of a successful two-hybrid interaction. These and other yeast colonies from the two-hybrid interaction of cDNA with NKX3.1 were restreaked on Leu$^-$, Trp$^-$, His$^-$, Ade$^-$ medium + X-gal to perform more stringent selection. The two-hybrid interaction is designed to activate three markers HIS, ADE and *LacZ*. The host yeast strain AH109 contains three marker genes ADE2, HIS3 and *LacZ*, each under the control of heterologous GAL4-responsive upstream activation sequences – GAL2, GAL1, and MEL1. This allowed us to test the two-hybrid reaction on three independent GAL4-responsive promoters and select only the clones with the strongest interaction.

After screening 2 x 10^5 cDNA clones we isolated 96 clones that gave rise to yeast strain AH109 growth in Leu$^-$, Trp$^-$, His$^-$ medium. Eighty-four of the 96 activated at least two of the three yeast reporter systems (His, Ade, *LacZ*); 51 clones activated all three reporters. A total of 66 of the original 96 clones were subjected to nucleotide sequencing. Five genes occurred more than once, but

Fig 9
Yeast Two-Hybrid Formation with NKX3.1



one of these, aminoacylase, which appeared twice, never gave positive *LacZ* colonies. The longest cDNA sequences of the three of the remaining clones were subjected to repeat two-hybrid interaction with NKX3.1. The results are shown in Table 1. The fourth clone, Na^+-K^+-dependent ATPase-β1, that was isolated three times, has yet to be analyzed.

Table 1
Two-Hybrid Confirmation of Clones Isolated >1 Time

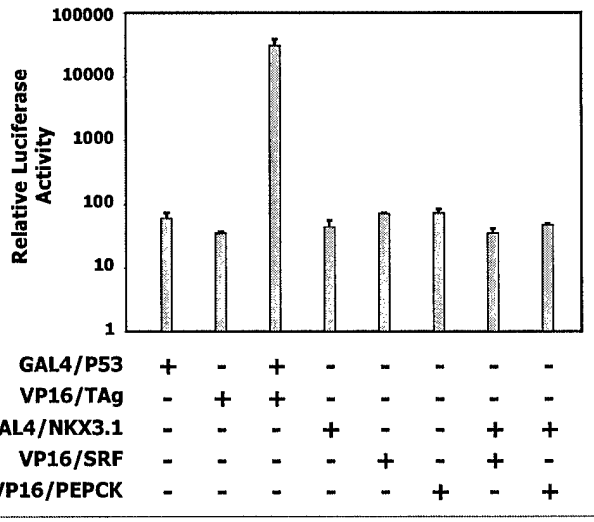
Clone	Gene	No. of clones	Colonies in Leu⁻, Trp⁻, His⁻		Colonies in Leu⁻, Trp⁻, His⁻, Ade⁻, LacZ+		
			AH109	AH109/NKX3.1	% His+	% Ade+	% *LacZ*+
CDNA-6	eEF1α[1]	4	0	1808	100	100	100
CDNA-55	MTHD[2]	2	0	1600	100	100	25
CDNA-72	PepCK	2	0	816	100	100	85
PGADT7	Neg control	N/A	0	0			

[1]Eukaryotic translation elongation factor1α
[2]Methylenetetrahydrofolate dehydrogenase-methylenetetrahydrofolate cyclohydrolase-formyltetrahydrofolate synthetase
[3]Phosphoenol pyruvate carboxykinase

A number of other clones were represented once in among the 66 clones sequenced. These included: ubiquitin protein ligase E3A, peroxisomal biogenesis factor 6, angio-associated migratory cell protein, sphingosine kinase type 2, glutathione peroxidase 3, and G-protein suppressor 2. Interestingly, a number of other metabolic enzymes were also isolated, for example glycine dehydrogenase, aldolase B, and glutaryl conenzyme A dehydrogenase.

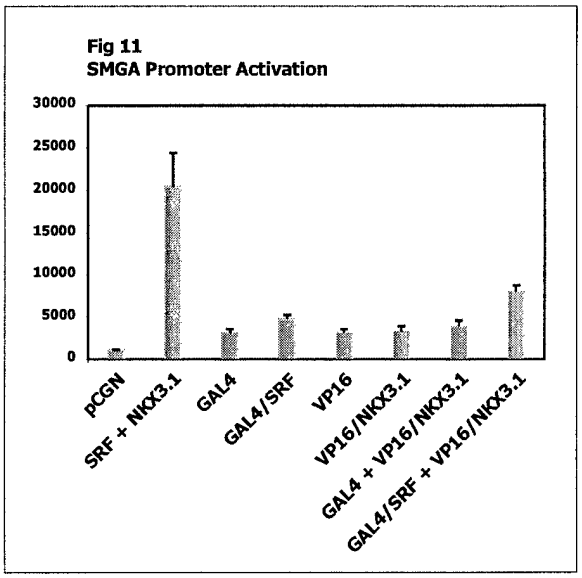
To confirm the binding results of the yeast two-hybrid experiments we subcloned NKX3.1 into mammalian two-hybrid vectors to create fusion proteins with GAL4 and with the Herpesvirus VP16 transcriptional activating protein. The generation of the correct fusion proteins from these vectors were confirmed by DNA sequencing and by western blotting. The full-length PepCK cDNA expression vector was obtained from K Jungermann (Goettingen, GE) [8]. This was cloned into the VP16 fusion vector to allow for mammalian two-hybrid testing by transcriptional activation of a GAL4-responsive reporter plasmid. The results of a representative mammalian two-hybrid experiment are shown in Figure 10. The binding of SV40 T-antigen (TAg) to P53 is shown on the left side of the figure and serves as a positive control for the assay. We were unable to demonstrate binding of NKX3.1 to SRF even though we previously had demonstrated physical association of NKX3.1 and SRF by coimmunoprecipitation and western blotting (Figure 11). This was of concern and was supposed to have provided a positive control for the binding ability of the GAL This was supposed to have provided a positive control for the binding ability of the

Fig 10
Mammalian Two-Hybrid Assay with NKX3.1

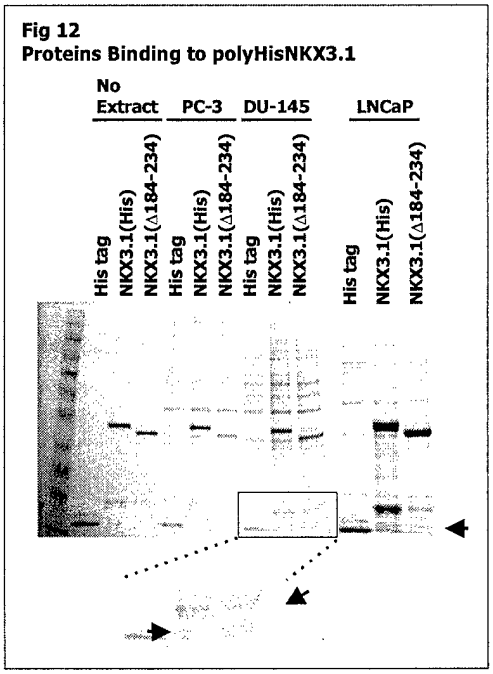


GAL4/NKX3.1 fusion protein. There was no evidence of binding of the GAL4/NKX3.1 fusion protein to either SRF or to PEPCK fusion proteins.

Because of the result shown in Figure 10, we wanted to determined whether the SRF and NKX3.1 fusion proteins retained biological activity as measured by the activation of the SMGA reporter. We constructed expression vectors for NKX3.1 fusion with VP16 and SRF fusion with GAL4. These constructs were tested in a reporter gene assay as shown in Figure 11. The positive control shown as the second result from the left is the interaction of the native NKX3.1 and SRF proteins. Note that the fusion constructs have no transcriptional activity. Because we were unable to show that the proteins assumed sufficient native conformation to mediate SMGA promoter activation, we concluded that this element of the Statement of Work cannot be accomplished and request the opportunity to replace this element with the tasks described below.

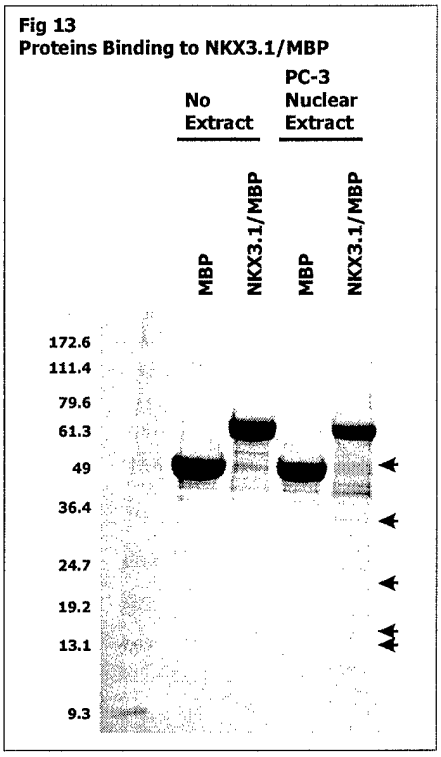


After extensive efforts at yeast two-hybrid cloning of NKX3.1 binding partners we have abandoned this approach due to artifacts resulting from GAL4/NKX3.1 fusion constructs. Although we considered pursuing LEXA fusions that we used by Chuck Bieberich to identify PDEF as an NKX3.1 binding protein[6], but after discussions with Chuck Bieberich we decided not to pursue these experiments. Instead we have decided to exploit affinity chromatography to isolate proteins that complex with NKX3.1. To generate preliminary data we exploited a large preparation of polyhistidylNKX3.1 that we are generating for structural studies. Polyhistidine, polyhistidylNKX3.1, and polyhistidylNKX3.1(Δ184-234) were each bound to nickel columns. The material bound to the columns in each case after extensive washing with low-salt buffer is shown in Figure 12 on the far left. We passed nuclear protein extracts from PC-3, DU-145, and LNCaP prostate cancer cells through the nickel columns as shown. The LNCaP cell extract was passed through a column that was loaded with more recombinant protein than the columns for PC-3 cells and DU-145 cells. In this preliminary experiment we observed a protein ~8KDa in DU-145 and LNCaP extracts that was uniquely retained by the polyhistidylNKX3.1 and polyhistidylNKX3.1(Δ184-234) (small arrows and magnified section of DU-145 lanes). This experiment demonstrates that in principle we can isolate proteins that bind to NKX3.1 using affinity chromatography.



We also tested the NKX3.1/MBP fusion protein as an affinity reagent. Figure 13 shows affinity columns made with MBP and NKX3.1/MBP. The two left lanes show the proteins from an

induced bacterial culture binding to an amylose column. The two right lanes show the proteins from PC-3 cell nuclear extracts that are retained on the columns. Note that the background retention on the MBP column is less than on the polyhistidine column making this a better substrate for initial separation of NKX3.1 binding proteins. Moreover, with this lower background it is easier to identify nuclear proteins that were retained specifically by the NKX3.1/MBP fusion protein (arrows).



C. Analysis of protein binding by NKX3.1 WT and mutant proteins. (Year 2-3)

1. Y2H assay with NKX3.1 WT, R52C and S48A in GAL4-DB constructs with SRF and other proteins in GAL4-AD constructs.

Will not be done as described above. We will replace this element with experiments that study the physical association of NKX3.1 with proteins identified by affinity binding.

2. GST pull down assays using NKX3.1 WT, R52C and S48A unphosphorylated and phosphorylated in vitro to bind to SRF and other binding partners.

No data to report.

3. Reporter gene assays using CMV promoter-luciferase and SMGA-luciferase reporters to assay activation by NKX3.1 WT, R52C and S48A with SRF and other binding proteins. Assay will be done in the presence and absence of 100 nM TPA.

No data to report.

D. Structural analysis of NKX3.1 – regions necessary for protein/protein interactions. (Year 3)

1. Construct GAL-4-DB vectors with *NKX3.1* deletion constructs.

No data to report.

2. Y2H analysis of deletion mutants binding to GAL4-AD-SRF and other protein binding partners.

Will not be done as described above. We will replace this element with experiments that study the physical association of NKX3.1 with proteins identified by affinity binding.

3. GST pull down assay using deletion mutants of NKX3.1-GST fusion protein and SRF or other cloned proteins

No data to report.

4. SMGA reporter gene analysis of transcriptional activation by *NKX3.1* deletion mutants and SRF. CMV reporter gene analysis of *NKX3.1* deletion mutants cotransfected with clones isolated in aim 2.

No data to report.

KEY RESEARCH ACCOMPLISHMENTS:

- Demonstration that both C- and N-terminal domains regulate SRF interaction of NKX3.1
- Identification of an NKX3.1-responsive region in the CMV promoter
- Demonstration of potential proteins that interact physically with NKX3.1

REPORTABLE OUTCOMES: None to date.

CONCLUSIONS:

This first year of the project has allowed us to demonstrate that regulation of NKX3.1 activity is governed by both N- and C-terminal domains of the protein. This finding is important because the critical region of the N-terminal domain includes the phosphorylation site near the genetic polymorphism at R52C. Further genetic analysis will elucidate the precise regions that govern the putative interaction of N- and C-terminal domains. We have also made progress in identifying a second activity of NKX3.1 that will allow us to assay the protein and discriminate between full-length and C-terminal truncated constructs. This activity is activation of the CMVΔ(1-3) promoter fragment. We have not as yet elucidated the precise mechanism of interaction between NKX3.1 and the CMVΔ(1-3) fragment. Further deletion analysis will allow us to focus on the DNA sequence responsible for the interaction and from there we may be able to identify candidate interacting transcription factors that mediate NKX3.1 effects on this promoter. We believe that the effect of NKX3.1 on CMVΔ(1-3) is one of coactivation because mutations that disrupt NKX3.1 DNA binding do not abrogate the CMV promoter activation.

We have also found that the yeast two-hybrid approach to selection of NKX3.1 binding proteins will not produce proteins that bind to NKX3.1 physiologically. Therefore we have decided to abandon this approach and all elements of the Statement of Work that rely on these experiments and instead approach the identification of NKX3.1 binding proteins by NKX3.1 affinity chromatography. Data was presented showing the feasibility of this approach.

The long-term goal of this work is to determine mechanisms to modulate NKX3.1 activity. This could lead to the identification of small molecules that can affect NKX3.1 activity and potentially modulate that activity in human cells. Since haploinsufficiency of NKX3.1 appears to be a gatekeeper event in prostate tumorigenesis, modulation of NKX3.1 activity may have a roll in prostate cancer treatment or prevention.

REFERENCES:

1. Voeller, H. J., Augustus, M., Madlike, V., Bova, G. S., Carter, K. C., and Gelmann, E. P. Coding region of NKX3.1, prostate-specific homeobox gene on 8p21, is not mutated in human prostate cancers. Cancer Res., *57*: 4455-4459, 1997.

2. Gelmann, E. P., Steadman, D. J., Ma, J., Ahronovitz, N., Voeller, H. J., Swope, S., Abbaszadegan, M., Brown, K. M., Strand, K., Hayes, R. B., and Stampfer, M. J. Occurrence of NKX3.1

C154T Polymorphism in Men with and without Prostate Cancer and Studies of Its Effect on Protein Function. Cancer Res., *62*: 2654-2659, 2002.

3. Steadman, D. J., Giuffrida, D., and Gelmann, E. P. DNA-binding sequence of the human prostate-specific homeodomain protein NKX3.1. Nucleic Acids Res., *28*: 2389-2395, 2000.

4. Chen, C. Y. and Schwartz, R. J. Recruitment of the tinman homolog Nkx-2.5 by serum response factor activates cardiac alpha-actin gene transcription. Mol.Cell Biol., *16*: 6372-6384, 1996.

5. Carson, J. A., Fillmore, R. A., Schwartz, R. J., and Zimmer, W. E. The Smooth Muscle gamma - Actin Gene Promoter Is a Molecular Target for the Mouse bagpipe Homologue, mNkx3-1, and Serum Response Factor. J.Biol.Chem., *275*: 39061-39072, 2000.

6. Chen, H., Nandi, A. K., Li, X., and Bieberich, C. J. NKX-3.1 interacts with prostate-derived Ets factor and regulates the activity of the PSA promoter. Cancer Res., *62*: 338-340, 2002.

7. Chen, C. Y., Croissant, J., Majesky, M., Topouzis, S., McQuinn, T., Frankovsky, M. J., and Schwartz, R. J. Activation of the cardiac alpha-actin promoter depends upon serum response factor, Tinman homologue, Nkx-2.5, and intact serum response elements. Dev.Genet., *19*: 119-130, 1996.

8. Modaressi, S., Christ, B., Bratke, J., Zahn, S., Heise, T., and Jungermann, K. Molecular cloning, sequencing and expression of the cDNA of the mitochondrial form of phosphoenolpyruvate carboxykinase from human liver. Biochem.J., *315 (Pt 3)*: 807-814, 1996.

APPENDICES:

Newly published paper:

Gelmann, E.P., Steadman, D.J., Ma, J., Ahronovitz, A., Voeller, H.J., Swope, S., Abbaszadegan, M., Brown, K., Strand, K., Hayes, R.B., and Stampfer, M.J. Occurrence of *NKX3.1* C154T polymorphism in men with and without prostate cancer and studies of its effect on protein function. *Cancer Res*, 62:2654-9, 2002.

[CANCER RESEARCH 62, 2654–2659, May 1, 2002]

Occurrence of *NKX3.1* C154T Polymorphism in Men with and without Prostate Cancer and Studies of Its Effect on Protein Function[1]

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ABSTRACT

NKX3.1, a member of the NK class of homeodomain proteins, is expressed primarily in the adult prostate and has growth suppression and differentiating effects in prostate epithelial cells. A C→T polymorphism at nucleotide 154 (*NKX3.1* C154T) is present in ~11% of healthy men with equal distribution among whites and blacks. In a cohort of 1253 prostate cancer patients and age-matched controls, the presence of the polymorphism was associated with a 1.8-fold risk of having stage C or D prostate cancer or Gleason score ≥7 (confidence interval, 1.01–3.22). The *NKX3.1* C154T polymorphism codes for a variant protein that contains an arginine-to-cysteine substitution at amino acid 52 (R52C) adjacent to a protein kinase C phosphorylation site at serine 48. Substitution of cysteine for arginine 52 or of alanine for serine 48 (S48A) reduced phosphorylation at serine 48 *in vitro* and *in vivo*. Phosphorylation of wild-type NKX3.1, but not of NKX3.1 R52C or NKX3.1 S48A, diminished binding *in vitro* to a high-affinity DNA binding sequence. NKX3.1 also serves as a transcriptional coactivator of serum response factor. Treatment of cells with 12-*O*-tetradecanoylphorbol-13-acetate to phosphorylate NKX3.1 had no effect on NKX3.1 coactivation of serum response factor. Neither the R52C nor the S48A substitution affected serum response factor coactivation by NKX3.1 We conclude that the polymorphic *NKX3.1* allele codes for a variant protein with altered DNA binding activity that may affect prostate cancer risk.

INTRODUCTION

Prostate cancer is a neoplasm with a variable natural history that ranges from indolent to aggressive. Low-grade or early-stage disease may have little impact on survival. However, patients with advanced stages or higher histological grades suffer substantial disease-related mortality (1). The occurrence of prostate cancer is influenced to a substantial degree by genetic factors (2, 3). Genetic determinants may affect individual risk for aggressive prostate cancer and, therefore, mortality from prostate cancer. For example, a polymorphic region in the androgen receptor gene affects the incidence of aggressive prostate cancer (4, 5).

NKX3.1 is an androgen-regulated NK-class homeobox gene with expression in adult mice and humans localized primarily in the prostate (6–9). The *NKX3.1* gene has been conserved during evolution; the murine and human proteins share 63% amino acid identity. The human *NKX3.1* has been mapped to chromosome 8p21 (10), a locus frequently deleted in prostate cancer (11–13). However, no tumor-specific mutations of the *NKX3.1* protein-coding region have been identified by genetic analysis of human prostate cancer samples (10). Nevertheless, loss of NKX3.1 expression was found in 6–15% of early-stage prostate cancer, 22% of locally advanced disease, 34% of hormone-refractory localized prostate cancer, and 78% of metastases (14). Decreased expression of NKX3.1 may have a role in prostate cancer pathogenesis because heterozygous *Nkx3.1* gene-targeted mice displayed a phenotype of prostatic hyperplasia, suggesting that *NKX3.1* haploinsufficiency may be dominant.

In the course of analyzing tumor samples for *NKX3.1* mutations, we found a C→T polymorphism at nucleotide 154 (C154T) that coded for a variant protein with a substitution of cysteine for arginine at amino acid 52 (R52C) of NKX3.1 (10). The polymorphism lay NH_2-terminal to the homeodomain in a region of the protein that was not conserved between mouse and human. We have determined the frequency of the polymorphism in a population of healthy men and examined its role as a possible risk factor for prostate cancer. We also show that the amino acid change coded by the polymorphism alters *in vitro* and *in vivo* properties of the protein.

MATERIALS AND METHODS

Plasmid Construction. Plasmids expressing full-length wild-type or polymorphic NKX3.1 fused to maltose-binding protein were generated as described previously (15). A plasmid encoding amino acids 1–184 (nucleotides 1–581) of wild-type NKX3.1 with an NH_2-terminal FLAG epitope was constructed. NKX3.1 point mutants were generated using a Quikchange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol. Mutant *NKX3.1* cDNAs were fully sequenced to confirm the presence of mutations and to ensure that no additional mutations were introduced.

Bacterial Expression and Purification of NKX3.1 Fusion Proteins. Plasmids expressing wild-type or NKX3.1 R52C were used to transform competent *Escherichia coli* strain BL21. The proteins were expressed and purified as described previously, using an amylose column that was eluted with 10 mM maltose (New England Biolabs, Beverly, MA; Ref. 15).

***In Vivo* Phosphorylation and Immunoprecipitation.** For labeling of exogenous NKX3.1, TSU-Pr1 or LNCaP cells were plated on a 6-cm dish in DMEM containing 5% fetal bovine serum (Life Technologies, Inc., Rockville, MD). At ~90% confluence, cells were transfected with 10 μg of wild-type or polymorphic *NKX3.1* expression vector or empty vector, using Lipofectamine 2000 according to the manufacturer's protocol (Life Technologies, Inc.). The *NKX3.1* constructs contained cDNA for expression of amino acids 1–184, including the NH_2 terminus and homeodomain of NKX3.1, because our data and those of others had shown that under the control of a cytomegalovirus promoter, the COOH-terminal truncated protein is expressed at higher levels than the wild-type protein (16).

Forty-eight h post-transfection, cells were labeled with 1 mCi/ml [^{32}P]P_i in carrier-free HCl (Amersham Pharmacia Biotech, Piscataway, NJ) for 3–4 h in phosphate-free DMEM containing 5% dialyzed fetal bovine serum (Life Technologies, Inc.). Labeling of endogenous NKX3.1 was done in LNCaP cells treated with 10 nM methyltrienolone (R1881; DuPont, Boston, MA). Cells were then treated with 100 nM TPA[3] (Sigma, St. Louis, MO) for 30 min before cell lysis. Labeled NKX3.1 was immunoprecipitated with either 1.5 μg of

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[3] The abbreviations used are: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; SRF, serum response factor; SMGA, smooth muscle γ-actin; FAM, 6-carboxyfluorescein; TET, 6-carboxy-4,7,2′,7′-tetrachlorofluorescein; TAMRA, 6-carboxy-*N*,*N*,*N*′,*N*′-tetramethylrhodamine.

anti-NKX3.1 polyclonal antiserum or 20 μg of anti-FLAG M2 antibody (Stratagene). Immunoprecipitates were electrophoresed on denaturing 10–20% gradient polyacrylamide gels followed by gel drying and autoradiography for visualization of radiolabeled proteins. Western blot analysis to determine protein levels was performed as described previously (14).

Phosphoamino Acid Analysis of NKX3.1. Labeled proteins were excised and eluted from polyacrylamide gels. The eluted protein was digested with 0.15 mg/ml trypsin overnight at 37°C, followed by hydrolysis with 1 ml of 6 N HCl at 105°C for 1 h. The HCl was removed by lyophilization, and the pellet was washed with 1 ml of H_2O and dried. Phosphoamino acids were separated by one-dimensional thin-layer electrophoresis as described previously (17). The identity of *in vivo* phosphorylated amino acids was determined by autoradiography followed by comparison of the autoradiogram with phosphoamino acid standards.

***In Vitro* Phosphorylation.** Synthetic peptides (30 μg) obtained from Research Genetics, Inc. (Huntsville, AL) or purified fusion proteins (200 ng) were incubated at 30°C for 30 min with 10 ng of a purified protein kinase C α, β, and γ isoform mixture (Upstate Biotechnology, Lake Placid, NY) in a buffer containing 20 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 0.2 mM EGTA, 5 mM DTT, 0.5 mM $CaCl_2$, 100 μg/ml phosphatidylserine, 50 μM ATP, and 0.11 μCi of [γ-^{32}P]ATP.

Electrophoretic Mobility Shift Assay. Gel shift assays were performed as described previously with modifications (15). Double-stranded DNA representing a consensus NKX3.1 binding site had the sequence 5′-GTATATA-AGTAGTTG-3′ (15).

Transcription Assay. CV-1 fibroblasts were maintained in Modified Improved MEM (Life Technologies, Inc.) supplemented with 5% fetal bovine serum. Cells were plated at ~1–2 × 10^5 cells/well in 12-well plates. Cells were transfected 24 h after plating, using Lipofectamine Plus according to the manufacturer's protocol (Life Technologies, Inc.). Each transfection reaction contained either 0.25 μg of SMGA reporter plasmid (a gift from Warren Zimmer, University of South Alabama (Mobile, AL); Ref. 16) or 0.2 μg of various NKX3.1 expression plasmids. SRF expression plasmid (0.5 μg; a gift from Ron Prywes, Columbia University, New York, NY; Ref. 18) was used as indicated. Total transfected DNA was always kept the same and balanced to 0.5 μg with empty vector. Cells were lysed 48 h after transfection, and the lysate was assayed for firefly luciferase activities with Dual Luciferase Reporter Assay Reagents (Promega, Madison, WI).

TaqMan Assay. The TaqMan allelic discrimination assay (19) was used to determine the frequency of the polymorphism at nucleotide 154 in prostate DNA samples. Genomic DNA was isolated using the Easy DNA Genomic DNA Isolation Kit (Invitrogen, Carlsbad, CA). The probe used to detect the wild-type codon was 5′-CAGAGACAGCGCGACCCGG-3′, and the probe used to detect the polymorphic codon was 5′-CAGAGACAGTGCGACCCG-GAGC-3′. The wild-type probe contained a 5′-FAM reporter dye, whereas the polymorphic probe had a 5′-TET reporter dye. Both probes had a 3′-TAMRA quencher dye. Probes used for allelic discrimination were synthesized by Biosearch Technologies, Inc. (Novato, CA). The forward primer used for PCR was 5′-CGCAGCGGCAAGGC-3′, and the reverse primer was 5′-GGTGCT-CAGCTGGTCGTTCT-3′ (Life Technologies, Inc., Rockville, MD). TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) was used in the PCR reaction according to the manufacturer's protocol. DNA (100 ng), primers (900 nM each), and probe (100 nM FAM-tagged or 200 nM TET-tagged) were added to the TaqMan Universal PCR Master Mix in a total volume of 50 μL. PCR was carried out on an ABI Prism 7700 Sequence Detection System (Applied Biosystems), using the following program: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 62°C for 1 min. Allelic discrimination analysis was performed on an ABI Prism 7700 Sequence Detection System (Applied Biosystems). When necessary, samples that contained the C154T polymorphism were confirmed by *Hha*I restriction digestion of PCR-amplified DNA. C154T abrogates a *Hha*I restriction endonuclease recognition site in the PCR transcript. The wild-type transcript was digested to 50-, 48-, and 21-nucleotide fragments, and the polymorphic allele to 98- and 21-nucleotide fragments.

Population Prevalence of *NKX3.1* (C154T). For the purpose of determining the frequency of *NKX3.1* (C154T) in different racial groups, *NKX3.1* genotype was assessed in a cohort of healthy male controls (n = 246; age range, 40–79 years) residing in Detroit, Michigan, or in 10 counties in New Jersey who had participated in a population-based case-control study (20). DNA was extracted from peripheral blood and assessed for *NKX3.1* genotype. Blood samples were obtained after informed consent. Cases from this case-control study were not included in the analysis.

Physicians Health Study Population. Blood samples were obtained in 1982 from 14,916 men enrolled in the Physicians Health Study. Follow-up questionnaires were completed by 99% of the men through 1995, and follow-up for vital status was 100%. Whenever prostate cancer was diagnosed in the cohort, we sought permission to obtain the medical records to determine stage at diagnosis, tumor grade, and Gleason score. If pathological staging was not available, the case was considered of indeterminate stage unless metastases were clinically evident. We categorized cases as high stage/grade if they were diagnosed at stages C or D, had a Gleason score ≥7, or had poor histological differentiation (4). We selected one or two controls at random for each case among the men who returned a blood specimen. Controls were men who had not undergone a radical prostatectomy, had not been diagnosed with prostate cancer at the time of the case diagnosis, and were matched by age and smoking status. DNA was extracted from peripheral blood and sent to E. G. for assay; all assays were performed blinded to case-control status. Samples from 558 cases and 695 controls were assayed. We calculated odds ratios as estimates for the relative risks and 95% confidence intervals from logistic regression models (21), controlling for the matching factors.

RESULTS

Occurrence of NKX3.1 R52C Variant in Prostate Cancer Patients and Healthy Men. To determine the frequency of the *NKX3.1* C154T polymorphism in the population, we tested DNA from healthy American white and black men. Prostate cancer incidence is substantially higher in black Americans than in whites, and prostate cancer deaths among blacks also exceed the rate in whites (22–24). We therefore wanted to determine whether there were racial differences in the occurrence of NKX3.1 R52C. We analyzed *NKX3.1* genotype in a cohort of 246 healthy men. Overall, 11% of men in the study population were found to carry the NKX3.1 R52C polymorphism. There was a no statistically significant difference in *NKX3.1* genotype distribution between the groups of white and black men (Table 1).

To ask whether *NKX3.1* genotype influenced prostate cancer risk, we analyzed a nested case-control study of 558 men with prostate cancer from the Physicians Health Study and 695 age-matched controls (4). The results for the total study population (Table 2) showed no statistically significant difference between the two groups. When we analyzed only men who presented with aggressive prostate cancer defined as stage C or D or Gleason score ≥7, we found a statistically significant increase in the frequency of NKX3.1 R52C among the cases (relative risk, 1.8; confidence interval, 1.01–3.22). No significant differences between cases and controls were found for nonaggressive cancers or for those men with unknown stage and grade. Because there appeared to be an effect of the polymorphic allele on prostate cancer risk, we sought to investigate whether the variant protein differed in activity from its wild-type counterpart.

Wild-Type and Variant NKX3.1 Are Differentially Phosphorylated by Protein Kinase C. The software program Phosphobase v2.0 was used to analyze the NKX3.1 amino acid sequence for possible phosphorylation sites (25). Three consensus sites were identified at serine 48 (44-GRTS*S*QRQR-52), threonine 117 (115-RLPQ*T*PKQP-123), and threonine 179 (175-RRYK*T*KRKRQ-184). Serine 48 was a candidate phosphorylation site for calmodulin kinase II, protein kinase

Table 1 *NKX3.1 genotype in white and black American men*

	NKX3.1 (genotype nt 154), *n* (%)			
	C/C	C/T	T/T	Total
White	116 (88%)	15	1	132
Black	103 (90%)	9	2	114
Total	219 (89%)	24	3	246

A, and protein kinase C. This site was of interest because it is located in close proximity to the NKX3.1 polymorphism at amino acid 52. Previously, Zannini *et al.* (26) showed that Nkx2.1 could be phosphorylated by protein kinase C. Wild-type NKX3.1 fused with maltose-binding protein was phosphorylated *in vitro* by protein kinase C (Fig. 1*A*). In addition to NKX3.1 phosphorylation, a minor level of protein kinase C autophosphorylation was present, represented by the 80-kDa band. Phosphobase v2.0 also identified four consensus protein kinase C phosphorylation sites in the amino acid sequence of the maltose-binding protein affinity tag. However, protein kinase C did not phosphorylate maltose-binding protein alone, suggesting that phosphorylation of the fusion protein was specific for the NKX3.1

Table 2 *Relative risk of prostate cancer according to the CGC→TGC polymorphism in NKX3.1*

NKX3.1 genotype	No. cases (%)	No. controls (%)	RR[a]	95% CI
Overall cancer				
CC	499 (89.4)	637 (91.7)	1.00	Reference
CT	57 (10.2)	55 (7.9)	1.32	0.90–1.95
TT	2 (0.4)	3 (0.4)	0.85	0.14–5.11
CT + TT			1.30	0.89–1.90
Nonaggressive cancer[b]				
CC	234 (90.4)	285 (89.6)	1.00	Reference
CT	25 (9.7)	31 (9.8)	0.98	0.56–1.71
TT	0	2 (0.6)	0	
CT + TT			0.92	0.53–1.60
Aggressive cancer[b]				
CC	229 (88.8)	313 (93.4)	1.00	Reference
CT	27 (10.5)	21 (6.3)	1.76	0.97–3.19
TT	2 (0.8)	1 (0.3)	2.73	0.25–30.33
CT + TT			1.80	1.01–3.22
Unknown aggressiveness				
CC	36 (87.8)	39 (92.9)	1.00	Reference
CT	5 (12.2)	3 (7.1)	1.81	0.40–8.10
TT	0	0	0	
CT + TT			1.81	0.40–8.10

[a] RR, relative risk; CI, confidence interval.

[b] Aggressive cancers were those cases identified at presentation as stages C or D or Gleason score ≥7.

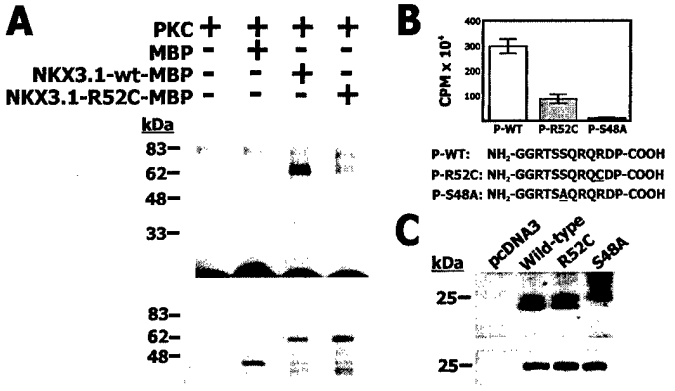

Fig. 1. Protein kinase C preferentially phosphorylates wild-type NKX3.1. *A*, purified maltose-binding protein (*MBP*) or NKX3.1 fusion proteins (200 ng) were used as substrates in kinase reactions with protein kinase C (10 ng) and 0.11 μCi of [γ-^{32}P]ATP. After the kinase reactions, samples were electrophoresed on a denaturing 10–20% gradient polyacrylamide gel. Phosphorylated proteins were identified by autoradiography (*top*). Western blotting with rabbit antiserum to maltose-binding protein (5 μg) was used to control for protein loading (*bottom*). *B*, peptides (30 μg) representing amino acids 43–54 of wild-type (*P-WT*), R52C (*P-R52C*), or S48A (*P-S48A*) NKX3.1 were used in an *in vitro* kinase assay with 10 ng of protein kinase C and 0.11 μCi of [γ-^{32}P]ATP. After the kinase reaction, samples were transferred to phosphocellulose discs and washed, and the incorporated radioactivity was measured as cpm by liquid scintillation counting. Amino acid sequences of the peptides are shown *below* the *graph*. *C*, LNCaP cells were transfected with vectors expressing wild-type, R52C, or S48A NKX3.1 with an NH_2-terminal FLAG tag. The cells were treated with R1881, and 48 h later, the cells were exposed to 1 mCi/ml [^{32}P]P_i. Cells were lysed, and NKX3.1 was immunoprecipitated with an anti-FLAG antibody. Immunoprecipitates were electrophoresed, and radiolabeled proteins were visualized by autoradiography (*top*). Western blotting with an anti-FLAG antibody (20 μg) was used to control for protein loading (*bottom*).

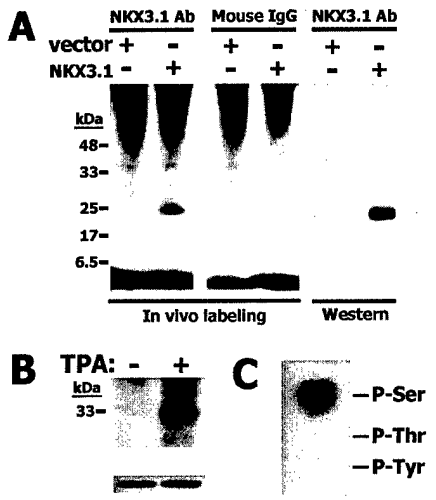

Fig. 2. NKX3.1 is phosphorylated *in vivo*. *A*, TSU-Pr1 cells were transfected with a wild-type NKX3.1 expression vector or empty vector. Forty-eight h post-transfection, cells were treated with 1 mCi/ml [^{32}P]P_i. Cells were then lysed, and NKX3.1 was immunoprecipitated with an anti-NKX3.1 antiserum or mouse IgG. Immunoprecipitates were electrophoresed by SDS-PAGE, and phosphorylated protein was visualized by autoradiography. The same antibody used for immunoprecipitation of NKX3.1 was used in a Western blot of lysates from cells transfected with either empty vector or NKX3.1 expression vector. *B*, LNCaP cells were treated with R1881, and 48 h later the cells were exposed to 1 mCi/ml [^{32}P]P_i. Cells were then treated with or without TPA (100 nM) for an additional 30 min. Cells were lysed, NKX3.1 was immunoprecipitated with an anti-NKX3.1 antibody and electrophoresed, and radiolabeled proteins were visualized by autoradiography (*top*). Western blotting with an anti-NKX3.1 antibody (1.5 μg) was used to control for protein loading (*bottom*). *C*, endogenous radiolabeled NKX3.1 was excised from a polyacrylamide gel, eluted, and treated with 0.15 mg/ml trypsin. The digested protein was hydrolyzed with 6 N HCl for 1 h at 105°C. Phosphoamino acids were separated by one-dimensional thin-layer electrophoresis. The identity of the phosphorylated amino acids was determined by autoradiography and comparison with phosphoamino acid standards.

moiety. In contrast, protein kinase C phosphorylation of NKX3.1 R52C was noticeably decreased relative to phosphorylation of wild-type NKX3.1 (Fig. 1*A*). The results of the Western blotting with anti-maltose-binding protein shown in the *bottom panel* of Fig. 1*A* indicate that equal amounts of fusion protein with either wild-type or NKX3.1 R52C were present in the reaction.

To confirm that the arginine-to-cysteine variation specifically affected phosphorylation by protein kinase C, synthetic peptide substrates representing amino acids 43–54 of NKX3.1 were used as protein kinase C substrates (Fig. 1*B*). Relative phosphorylation of peptide from wild-type NKX3.1 was 3-fold higher than for the NKX3.1 R52C peptide. Phosphorylation of peptide with an alanine replacing serine 48 was decreased 33-fold relative to the wild-type sequence. To determine the effects of amino acid alterations on the serine 48 phosphorylation *in vivo*, NKX3.1, NKX3.1 R52C, or NKX3.1 S48A expression vectors with a FLAG tag were used to transfect LNCaP prostate cancer cells that expressed endogenous NKX3.1 as well (7). The level of phosphorylation in the cells transfected with the NKX3.1 R52C variant was half that in the cells transfected with the wild-type protein. The mutation of serine 48 to alanine essentially eliminated *in vivo* phosphorylation of Flag-tagged NKX3.1 (Fig. 1*C*), providing evidence that serine 48 is a major *in vivo* phosphoacceptor.

TSU-Pr1 cells, which do not express NKX3.1, were used for [^{32}P]P_i labeling of COOH-terminal truncated NKX3.1 (Fig. 2*A*). Western blotting confirmed that only exogenous NKX3.1 protein was detected in the cells (Fig. 2*A*). Endogenous full-length NKX3.1 in LNCaP cells was also phosphorylated *in vivo*, and the level of phosphorylation was increased by the presence of 100 nM TPA (Fig. 2*B*), suggesting that NKX3.1 was phosphorylated *in vivo* by a TPA-induced kinase, such as protein kinase C. Protein kinase C did not affect levels of endog-

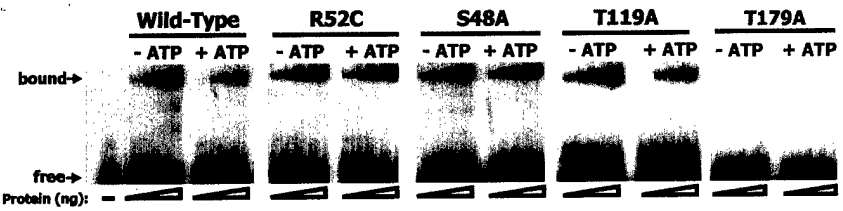

Fig. 3. R52C polymorphism affects phosphorylation-regulated DNA binding. Purified fusion proteins (200 ng) were treated with protein kinase C (10 ng) in the presence or absence of cold ATP. After protein kinase C treatment, the proteins (2, 5, 10, or 25 ng) were used in gel shift assays with a radiolabeled NKX3.1 consensus DNA binding sequence. Protein-bound DNA was separated from free probe by 8% native PAGE, and the results were visualized by autoradiography.

enous NKX3.1 as determined by Western blotting (Fig. 2*B*). Phosphoamino acid analysis of the radiolabeled endogenous protein in LNCaP cells indicated that NKX3.1 was phosphorylated only at serine (Fig. 2*C*). Similar phosphoamino acid analysis results were obtained when we labeled exogenous NKX3.1 in transfected TSU-Pr1 cells (data not shown). Moreover, no phosphorylation was seen in several attempts to label NKX3.1 S48A *in vivo*.

Phosphorylation at Serine 48 Regulates *in Vitro* NKX3.1 DNA Binding. Recombinant purified NKX3.1 fusion proteins were treated with protein kinase C in the presence or absence of ATP, and the proteins were included in gel shift assays with a radiolabeled NKX3.1 high-affinity DNA binding sequence (15). Phosphorylation of wild-type NKX3.1 decreased the apparent binding affinity of the protein for the consensus sequence by 3-fold relative to the nonphosphorylated protein (Fig. 3). However, the DNA binding of NKX3.1 R52C was not noticeably altered after treatment with protein kinase C in the presence of ATP. Similarly, NKX3.1 S48A lost regulation of DNA binding by phosphorylation (Fig. 3). To compare the effects of phosphorylation at two other consensus protein kinase C phosphorylation sites on DNA binding, either threonine 119 or threonine 179 was mutated to alanine. Protein kinase C treatment of NKX3.1 T119A yielded DNA binding data similar to those for wild-type NKX3.1 (Fig. 3). Interestingly, the T179A mutation, located in the homeodomain, abrogated NKX3.1 DNA binding (Fig. 3).

Effect of Polymorphism on NKX3.1 Transcriptional Activity. NKX3.1 and the heart-specific NK family protein NKX2.5 have very similar *in vitro* properties. The activities of these proteins are mediated largely by the homeodomains, which are nearly identical in their three major homeodomain helices and coincide at 39 of 60 amino acids. Both proteins bind SRF (16, 27), and NKX2.5 was shown to bind SRF *via* the homeodomain (27). Because of similarities in their homeodomain primary structure, NKX3.1 is likely also to bind SRF via the homeodomain. To assess NKX3.1 coactivation of SRF, we used a transcription assay with a reporter construct under control of the SMGA promoter, similar to the transactivation experiment reported by Carson *et al.* (16). We found that human NKX3.1 can act as a coactivator for SRF activation of transcription from the SMGA promoter, similar to previously published results for murine Nkx3.1 (16). In general, the presence of NKX3.1 resulted in a 3–5-fold increase in SMGA promoter activity. We compared full-length wild-type NKX3.1 expression vector with mutant constructs that coded for NKX3.1 R52C and NKX3.1 S48A protein variants. As a control we used an expression construct with *NKX3.1* in reverse orientation that coded for no protein. The three coding constructs had similar levels of NKX3.1 protein expression and similar levels of SRF coactivation. Treatment of the cultures with 100 nM TPA, which had been shown to cause phosphorylation of NKX3.1 in culture, had a minimal inhibitory effect on the activity of each construct, but did not differentially affect the coactivation by mutant and wild-type constructs (Table 3).

DISCUSSION

A common polymorphism in the prostate-specific homeoprotein may have an affect on prostate cancer pathogenesis as risk factor for aggressive disease. A tumor suppressor function of NKX3.1 has been suggested by studies of gene-targeted mice. Targeted disruption of murine *Nkx3.1* suggested that the gene exerts growth suppression and differentiating effects on prostatic epithelium (9, 28). Importantly, animals heterozygous for loss of Nkx3.1 demonstrated histological disarray of the prostate and bulbourethral gland, suggesting that haploinsufficiency was dominant. Because the murine gene lacks an amino acid similarity at arginine 52 and lacks the protein kinase C phosphorylation site at serine 48, it is hard to predict the role of this putative regulatory region in the mouse.

We found that the R52C polymorphism occurs with similar frequency among whites and blacks in the United States. Prostate cancer is more common among blacks than whites in the United States, with a higher mortality among blacks than whites (22–24). Therefore, it does not appear that disparities in the frequency of *NKX3.1* C154T contribute to the difference in prostate cancer between the races. Approximately 5–10% of prostate cancer is inherited in a Mendelian fashion that has been traced to at least three susceptibility loci on chromosomes 1, X, and 17 (29–32).

The occurrence of sporadic prostate cancer, however, is likely to be influenced subtly by many genes that affect susceptibility. Much attention has been directed to variations in the polyglutamine tract in the NH_2 terminus of the androgen receptor. Shorter polyglutamine repeat lengths are associated with increased androgen receptor activity and more aggressive prostate cancer (4, 5, 33). Other genetic factors that may have a subtle effect on prostate cancer risk in the general population include the vitamin D receptor (34–36), *CYP17* (37), 5α-reductase A49T (38), and glutathione *S*-transferase θ (39). The *NKX3.1* C154T genotype may be one of those subtle genetic influences on prostate cancer risk, in particular for aggressive disease.

The *NKX3.1* C154T polymorphism appears to affect a region of the protein that can affect DNA binding. The exact role of the region containing amino acids 48–52 has not been determined, but it is clear that the region is important for phosphorylation. Homeoproteins are known to undergo posttranslational modification by phosphorylation. Homeoprotein phosphorylation has been shown to affect protein-protein interactions (40), subcellular localization (41), DNA binding affinity (42), and transcriptional activity (43). Generally, these effects

Table 3 *SRF coactivation assay of NKX3.1 and mutant constructs*

Construct	Fold SRF coactivation	
	−TPA	+TPA
Control	1.06	NT[a]
NXK3.1	4.4	3.7
NKX3.1 R52C	5.8	4.6
NKX3.1 S48A	6.8	5.6

[a] NT, not tested.

have been attributed to electrostatic repulsion or a conformational change in the protein (44). Members of the NK family of homeoproteins have been shown to undergo phosphorylation. The kinases responsible for phosphorylating NK-class homeoproteins include casein kinase II (43), MST2 kinase (45), extracellular signal-regulated kinase (46), homeodomain-interacting protein kinase (47), protein kinase A (48), and protein kinase C (26).

Although we believe that the cellular activity of NKX3.1 R52C is different from that of the wild type, the precise impact of the polymorphism on NKX3.1 function is unclear. Although relatively little is known about the protein interactions of NKX3.1, the NK family member NKX2.5 has been characterized more extensively and has been shown to interact with DNA and with at least two other transcription factors, GATA-4 and SRF (27, 49, 50). Moreover, the protein-protein interactions of NKX3.1 are mediated by the homeodomain as well (27, 49, 50). It is entirely possible that NKX3.1, like NKX2.5, undergoes multiple interactions that are involved in manifestation of its biological effects. However, the analogy between the two homeoproteins has yet to be proved. It should be remembered that although the two proteins share nearly identical homeodomain sequences, they have very little amino acid identity in the NH_2- and COOH-terminal regions.

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REFERENCES

1. Albertsen, P. C., Hanley, J. A., Gleason, D. F., and Barry, M. J. Competing risk analysis of men aged 55 to 74 years at diagnosis managed conservatively for clinically localized prostate cancer. JAMA, *280:* 975–980, 1998.
2. Lichtenstein, P., Holm, N. V., Verkasalo, P. K., Iliadou, A., Kaprio, J., Koskenvuo, M., Pukkala, E., Skytthe, A., and Hemminki, K. Environmental and heritable factors in the causation of cancer—analyses of cohorts of twins from Sweden. Denmark, and Finland. N. Engl. J. Med., *343:* 78–85, 2000.
3. Steinberg, G. D., Carter, B. S., Beaty, T. H., Childs, B., and Walsh, P. C. Family history and the risk of prostate cancer. Prostate, *17:* 337–347, 1990.
4. Giovannucci, E., Stampfer, M. J., Krithivas, K., Brown, M., Brufsky, A., Talcott, J., Hennekens, C. H., and Kantoff, P. W. The CAG repeat within the androgen receptor gene and its relationship to prostate cancer. Proc. Natl. Acad. Sci., USA, *94:* 3320–3323, 1997.
5. Hsing, A. W., Gao, Y. T., Wu, G., Wang, X., Deng, J., Chen, Y. L., Sesterhenn, I. A., Mostofi, F. K., Benichou, J., and Chang, C. Polymorphic CAG and GGN repeat lengths in the androgen receptor gene and prostate cancer risk: a population-based case-control study in China. Cancer Res, *60:* 5111–5116, 2000.
6. Kim, Y., and Nirenberg, M. *Drosophila* NK-homeobox genes. Proc. Natl. Acad. Sci. USA, *86:* 7716–7720, 1989.
7. He, W. W., Sciavolino, P. J., Wing, J., Augustus, M., Hudson, P., Meissner, P. S., Curtis, R. T., Shell, B. K., Bostwick, D. G., Tindall, D. J., Gelmann, E. P., Abate-Shen, C., and Carter, K. C. A novel human prostate-specific, androgen-regulated homeobox gene (*NKX3.1*) that maps to 8p21, a region frequently deleted in prostate cancer. Genomics, *43:* 69–77, 1997.
8. Prescott, J. L., Blok, L., and Tindall, D. J. Isolation and androgen regulation of the human homeobox cDNA NKX3.1. Prostate, *35:* 71–80, 1998.
9. Bhatia-Gaur, R., Donjacour, A. A., Sciavolino, P. J., Kim, M., Desai, N., Norton, C. R., Gridley, T., Cardiff, R. D., Cunha, G. R., Abate-Shen, C., and Shen, M. M. Roles for Nkx3.1 in prostate development and cancer. Genes Dev., *13:* 966–977, 1999.
10. Voeller, H. J., Augustus, M., Madlike, V., Bova, G. S., Carter, K. C., and Gelmann, E. P. Coding region of NKX3.1, prostate-specific homeobox gene on 8p21, is not mutated in human prostate cancers. Cancer Res., *57:* 4455–4459, 1997.
11. Lundgren, R., Kristoffersson, U., Heim, S., Mandahl, N., and Mitelman, F. Multiple structural chromosome rearrangements, including del(7q) and del(10q), in an adenocarcinoma of the prostate. Cancer Genet. Cytogenet., *35:* 103–108, 1988.
12. Carter, B. S., Ewing, C. M., Ward, W. S., Treiger, B. F., Aalders, T. W., Schalken, J. A., Epstein, J. I., and Isaacs, W. B. Allelic loss of chromosomes 16q and 10q in human prostate cancer. Proc. Natl. Acad. Sci. USA, *87:* 8751–8755, 1990.
13. Phillips, S. M., Morton, D. G., Lee, S. J., Wallace, D. M., and Neoptolemos, J. P. Loss of heterozygosity of the retinoblastoma and adenomatous polyposis susceptibility gene loci and in chromosomes 10p, 10q and 16q in human prostate cancer. Br. J. Urol., *73:* 390–395, 1994.
14. Bowen, C., Bubendorf, L., Voeller, H. J., Slack, R., Willi, N., Sauter, G., Gasser, T. C., Koivisto, P., Lack, E. E., Kononen, J., Kallioniemi, O. P., and Gelmann, E. P. Loss of NKX3.1 expression in human prostate cancers correlates with tumor progression. Cancer Res., *60:* 6111–6115, 2000.
15. Steadman, D. J., Giuffrida, D., and Gelmann, E. P. DNA-binding sequence of the human prostate-specific homeodomain protein NKX3.1. Nucleic Acids Res., *28:* 2389–2395, 2000.
16. Carson, J. A., Fillmore, R. A., Schwartz, R. J., and Zimmer, W. E. The smooth muscle γ-actin gene promoter is a molecular target for the mouse bagpipe homologue, mNkx3–1, and serum response factor. J. Biol. Chem., *275:* 39061–39072, 2000.
17. Hirano, A. A., Greengard, P., and Huganir, R. L. Protein tyrosine kinase activity and its endogenous substrates in rat brain: a subcellular and regional survey. J. Neurochem., *50:* 1447–1455, 1988.
18. Prywes, R., Dutta, A., Cromlish, J. A., and Roeder, R. G. Phosphorylation of serum response factor, a factor that binds to the serum response element of the c-FOS enhancer. Proc Natl. Acad. Sci. USA, *85:* 7206–7210, 1988.
19. Heid, C. A., Stevens, J., Livak, K. J., and Williams, P. M. Real time quantitative PCR. Genome Res., *6:* 986–994, 1996.
20. Hayes, R. B., Pottern, L. M., Strickler, H., Rabkin, C., Pope, V., Swanson, G. M., Greenberg, R. S., Schoenberg, J. B., Liff, J., Schwartz, A. G., Hoover, R. N., and Fraumeni, J. F., Jr. Sexual behaviour, STDs and risks for prostate cancer. Br. J. Cancer, *82:* 718–725, 2000.
21. Kleinbaum, D. G., Kupper, L. L., and Morgenstern, H. Epidemiologic Research: Principles and Quantitative Methods. New York: Van Nostrand Reinhold, 1982.
22. Stanford, J. L., Stephenson, R. A., Coyle, L. M., Cerhan, J., Correa, R., Eley, J. W., Gilliland, F., Hankey, B., Kolonel, L. N., Kosary, C. L., Ross, R., Severson, R., and West, D. Prostate Cancer Trends, 1973–1995, SEER Program. Bethesda, MD: National Cancer Institute, 1998.
23. Feuer, E. J., Merrill, R. M., and Hankey, B. F. Cancer surveillance series: interpreting trends in prostate cancer—part II: cause of death misclassification and the recent rise and fall in prostate cancer mortality. J. Natl. Cancer Inst. (Bethesda), *91:* 1025–1032, 1999.
24. Hankey, B. F., Feuer, E. J., Clegg, L. X., Hayes, R. B., Legler, J. M., Prorok, P. C., Ries, L. A., Merrill, R. M., and Kaplan, R. S. Cancer surveillance series: interpreting trends in prostate cancer—part I: evidence of the effects of screening in recent prostate cancer incidence, mortality, and survival rates. J. Natl. Cancer Inst. (Bethesda), *91:* 1017–1024, 1999.
25. Kreegipuu, A., Blom, N., and Brunak, S. PhosphoBase, a database of phosphorylation sites: release 2.0. Nucleic Acids Res., *27:* 237–239, 1999.
26. Zannini, M., Acebron, A., De Felice, M., Arnone, M. I., Martin-Perez, J., Santisteban, P., and Di Lauro, R. Mapping and functional role of phosphorylation sites in the thyroid transcription factor-1 (TTF-1). J. Biol. Chem., *271:* 2249–2254, 1996.
27. Chen, C. Y., and Schwartz, R. J. Recruitment of the tinman homolog Nkx-2.5 by serum response factor activates cardiac α-actin gene transcription. Mol. Cell. Biol., *16:* 6372–6384, 1996.
28. Schneider, A., Brand, T., Zweigerdt, R., and Arnold, H. Targeted disruption of the nkx3.1 gene in mice results in morphogenetic defects of minor salivary glands: parallels to glandular duct morphogenesis in prostate. Mech. Dev., *95:* 163–174, 2000.
29. Carter, B. S., Bova, G. S., Beaty, T. H., Steinberg, G. D., Childs, B., Isaacs, W. B., and Walsh, P. C. Hereditary prostate cancer. Epidemiology and clinical features. J. Urol., *150:* 797–802, 1993.
30. Smith, J. R., Freije, D., Carpten, J. D., Gronberg, H., Xu, J., Isaacs, S. D., Brownstein, M. J., Bova, G. S., Guo, H., Bujnovszky, P., Nusskern, D. R., Damber, J. E., Bergh, A., Emanuelsson, M., Kallioniemi, O. P., Walker-Daniels, J., Bailey-Wilson, J. E., Beaty, T. H., Meyers, D. A., Walsh, P. C., Collins, F. S., Trent, J. M., and Isaacs, W. B. Major susceptibility locus for prostate cancer on chromosome 1 suggested by a genome-wide search. Science (Wash. DC), *274:* 1371–1374, 1996.
31. Xu, J., Meyers, D., Freije, D., Isaacs, S., Wiley, K., Nusskern, D., Ewing, C., Wilkens, E., Bujnovszky, P., Bova, G. S., Walsh, P., Isaacs, W., Schleutker, J., Matikainen, M., Tammela, T., Visakorpi, T., Kallioniemi, O. P., Berry, R., Schaid, D., French, A., McDonnell, S., Schroeder, J., Blute, M., Thibodeau, S., and Trent, J. Evidence for a prostate cancer susceptibility locus on the X chromosome. Nat. Genet., *20:* 175–179, 1998.
32. Tavtigian, S. V., Simard, J., Teng, D. H., Abtin, V., Baumgard, M., Beck, A., Camp, N. J., Carillo, A. R., Chen, Y., Dayananth, P., Desrochers, M., Dumont, M., Farnham, J. M., Frank, D., Frye, C., Ghaffari, S., Gupte, J. S., Hu, R., Iliev, D., Janecki, T., Kort, E. N., Laity, K. E., Leavitt, A., Leblanc, G., McArthur-Morrison, J., Pederson, A., Penn, B., Peterson, K. T., Reid, J. E., Richards, S., Schroeder, M., Smith, R., Snyder, S. C., Swedlund, B., Swensen, J., Thomas, A., Tranchant, M., Woodland, A. M., Labrie, F., Skolnick, M. H., Neuhausen, S., Rommens, J., and Cannon-Albright, L. A. A candidate prostate cancer susceptibility gene at chromosome 17p. Nat. Genet., *27:* 172–180, 2001.
33. Beilin, J., Ball, E. M., Favaloro, J. M., and Zajac, J. D. Effect of the androgen receptor CAG repeat polymorphism on transcriptional activity: specificity in prostate and non-prostate cell lines. J. Mol. Endocrinol., *25:* 85–96, 2000.
34. Correa-Cerro, L., Berthon, P., Haussler, J., Bochum, S., Drelon, E., Mangin, P., Fournier, G., Paiss, T., Cussenot, O., and Vogel, W. Vitamin D receptor polymorphisms as markers in prostate cancer. Hum. Genet., *105:* 281–287, 1999.
35. Ingles, S. A., Ross, R. K., Yu, M. C., Irvine, R. A., La Pera, G., Haile, R. W., and Coetzee, G. A. Association of prostate cancer risk with genetic polymorphisms in vitamin D receptor and androgen receptor. J. Natl. Cancer Inst. (Bethesda), *89:* 166–170, 1997.
36. Taylor, J. A., Hirvonen, A., Watson, M., Pittman, G., Mohler, J. L., and Bell, D. A. Association of prostate cancer with vitamin D receptor gene polymorphism. Cancer Res., *56:* 4108–4110, 1996.

37. Gsur, A., Bernhofer, G., Hinteregger, S., Haidinger, G., Schatzl, G., Madersbacher, S., Marberger, M., Vutuc, C., and Micksche, M. A polymorphism in the *CYP17* gene is associated with prostate cancer risk. Int. J. Cancer, *87:* 434–437, 2000.
38. Makridakis, N. M., Ross, R. K., Pike, M. C., Crocitto, L. E., Kolonel, L. N., Pearce, C. L., Henderson, B. E., and Reichardt, J. K. Association of mis-sense substitution in *SRD5A2* gene with prostate cancer in African-American and Hispanic men in Los Angeles, USA. Lancet, *354:* 975–978, 1999.
39. Rebbeck, T. R., Walker, A. H., Jaffe, J. M., White, D. L., Wein, A. J., and Malkowicz, S. B. Glutathione *S*-transferase-μ (GSTM1) and -θ (GSTT1) genotypes in the etiology of prostate cancer. Cancer Epidemiol. Biomark. Prev., *8:* 283–287, 1999.
40. Li, C., and Manley, J. L. Allosteric regulation of even-skipped repression activity by phosphorylation. Mol. Cell, *3:* 77–86, 1999.
41. Moede, T., Leibiger, B., Pour, H. G., Berggren, P., and Leibiger, I. B. Identification of a nuclear localization signal, RRMKWKK, in the homeodomain transcription factor PDX-1. FEBS Lett., *461:* 229–234, 1999.
42. Bourbon, H. M., Martin-Blanco, E., Rosen, D., and Kornberg, T. B. Phosphorylation of the *Drosophila* engrailed protein at a site outside its homeodomain enhances DNA binding. J. Biol. Chem., *270:* 11130–11139, 1995.
43. Kasahara, H., and Izumo, S. Identification of the *in vivo* casein kinase II phosphorylation site within the homeodomain of the cardiac tissue-specifying homeobox gene product Csx/Nkx2.5. Mol. Cell. Biol., *19:* 526–536, 1999.
44. Karin, M. Signal transduction from the cell surface to the nucleus through the phosphorylation of transcription factors. Curr. Opin. Cell Biol., *6:* 415–424, 1994.
45. Aurisicchio, L., Di Lauro, R., and Zannini, M. Identification of the thyroid transcription factor-1 as a target for rat MST2 kinase. J. Biol. Chem., *273:* 1477–1482, 1998.
46. Missero, C., Pirro, M. T., and Di Lauro, R. Multiple ras downstream pathways mediate functional repression of the homeobox gene product TTF-1. Mol. Cell. Biol., *20:* 2783–2793, 2000.
47. Kim, Y. H., Choi, C. Y., Lee, S. J., Conti, M. A., and Kim, Y. Homeodomain-interacting protein kinases, a novel family of co-repressors for homeodomain transcription factors. J. Biol. Chem., *273:* 25875–25879, 1998.
48. Yan, C., and Whitsett, J. A. Protein kinase A activation of the surfactant protein B gene is mediated by phosphorylation of thyroid transcription factor 1. J. Biol. Chem., *272:* 17327–17332, 1997.
49. Sepulveda, J. L., Belaguli, N., Nigam, V., Chen, C. Y., Nemer, M., and Schwartz, R. J. GATA-4 and Nkx-2.5 coactivate Nkx-2 DNA binding targets: role for regulating early cardiac gene expression. Mol. Cell. Biol., *18:* 3405–3415, 1998.
50. Chen, C. Y., Croissant, J., Majesky, M., Topouzis, S., McQuinn, T., Frankovsky, M. J., and Schwartz, R. J. Activation of the cardiac α-actin promoter depends upon serum response factor, Tinman homologue, Nkx-2.5, and intact serum response elements. Dev. Genet., *19:* 119–130, 1996.